Deletion of Myeloid GSK3α Attenuates Atherosclerosis and Promotes an M2 Macrophage Phenotype

Cameron S. McAlpine, Aric Huang, Abby Emdin, Nicole S. Banko, Daniel R. Beriault, Yuanyuan Shi, Geoff H. Werstuck

Objective—Glycogen synthase kinase (GSK)-3α/β has been implicated in the pathogenesis of diabetes mellitus, cancer, Alzheimer, and atherosclerosis. The tissue- and homolog-specific functions of GSK3α and β in atherosclerosis are unknown. This study examines the effect of hepatocyte or myeloid cell deletion of GSK3α or GSK3β on atherosclerosis in low-density lipoprotein receptor (LDLR)−/− mice.

Approach and Results—We ablated GSK3α or GSK3β expression in hepatic or myeloid cells of LDLR−/− mice, and mice were fed a high-fat diet for 10 weeks. GSK3α or GSK3β deficiency in hepatic or myeloid cells did not affect metabolic parameters, including plasma lipid levels. Hepatic deletion of GSK3α or GSK3β did not affect the development of atherosclerosis or hepatic lipid content. Myeloid deletion of GSK3α, but not of GSK3β, reduced atherosclerotic lesion volume and lesion complexity. Mice lacking GSK3α in myeloid cells had a less inflammatory and more anti-inflammatory plasma cytokine profile. Macrophages within atherosclerotic lesions of myeloid GSK3α-deficient mice, but not of GSK3β-deficient mice, displayed reduced expression of markers associated with M1 macrophage polarization and enhanced expression of the M2 markers. Finally, bone marrow–derived macrophages were isolated and differentiated into classical M1 macrophages or alternative M2 macrophages in vitro. GSK3α deletion, but not GSK3β deletion, attenuated the expression of genes associated with M1 polarization while promoting the expression of genes associated with M2 polarization by modulating STAT3 and STAT6 activation.

Conclusions—Our findings suggest that deletion of myeloid GSK3α attenuates the progression of atherosclerosis by promoting an M2 macrophage phenotype. (Arterioscler Thromb Vasc Biol. 2015;35:00:00. DOI: 10.1161/ATVB.AHA.115.305438.)

Key Words: atherosclerosis • glycogen synthase kinase 3

Atherosclerosis is the primary underlying pathology of cardiovascular disease, which accounts for the majority of deaths in developed nations.1 Myeloid lineage cells are critical mediators in the development of atherosclerosis and account for the majority of a lesion’s cellular bulk.2 Within the atherosclerotic lesion, macrophages phagocytose modified lipid particles becoming lipid-engorged foam cells. Foam cells exacerbate disease progression through the secretion of proinflammatory cytokines and growth factors. In advanced lesions, foam cells undergo apoptosis leading to the formation of a lipid-rich, acellular, and highly thrombotic necrotic core. The underlying molecular mechanisms that regulate myeloid cell behavior during atherosclerosis remain poorly defined.

Increasing evidence suggests that myeloid cell subpopulations are heterogeneous and have distinctive phenotypes that play unique roles in disease states. Macrophages are often broadly classified as having a classical (M1) or an alternative (M2) phenotype. M1 macrophages, elicited by toll-like receptor or interferon-γ receptor stimulation, are the most prominent macrophages at sites of inflammation and exacerbate the inflammatory response through the secretion of proinflammatory cytokines and chemoattractants.3,4 M1 macrophages promote atherosclerotic lesion development and complexity.5–7 M2 macrophages patrol tissue, perform reparative and immunoregulatory functions, efferocytose debris, and are antiatherogenic.3,4,8 Other macrophage subtypes have been identified in atherosclerotic lesions, including M4, Mox, and Mhem; however, their roles are less well characterized.5,9,10

Our understanding of the cellular signaling networks that regulate macrophage polarization in the context of atherosclerosis and the relative contribution of each phenotype to the progression and development of atherosclerosis is limited.

Glycogen synthase kinase (GSK)-3 α and β are homologous serine/threonine kinases encoded by separate genes.11 GSK3α and GSK3β share 98% amino acid homology within their kinase domain but only 36% homology in the C-terminal domain. GSK3α (51 kDa) is 5 kDa larger than GSK3β (46 kDa) because an N-terminal glycine-rich domain with an

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unknown function. Both GSK3α and GSK3β are ubiquitously expressed although expression levels vary greatly from tissue to tissue. GSK3α/β is found in the cytoplasm, endoplasmic reticulum, and nucleus of most cells. Originally described as a regulator of glycogen synthase and β-catenin, GSK3β is now recognized to be a target of several signaling networks, including insulin/PI3K/AKT, MAPKs, and endoplasmic reticulum stress. Although GSK3α and GSK3β share several common substrates, they are not redundant and seem to have distinct functions.

Whole-body GSK3α-deficient mice are viable and develop normally, whereas GSK3β deletion is embryonically lethal. Recent evidence has suggested a role of GSK3α/β in atherosclerosis. In mouse models, pharmacological inhibition of GSK3α/β or whole-body GSK3β deletion attenuates atherosclerotic lesion formation and the development of hepatic steatosis. Although suggestive of a role for GSK3α/β in atherosclerosis, the homolog- and tissue-specific functions of GSK3α and GSK3β, as well as the mechanism by which GSK3α/β regulates atherosclerosis, are not known.

We have developed hepatocyte and myeloid cell–specific GSK3α or GSK3β knockout low-density lipoprotein receptor (LDLR)−/− mice. Our results demonstrate that GSK3α and GSK3β play distinctive homolog- and tissue-specific roles during atherogenesis. Specifically, myeloid GSK3α, but not GSK3β, deletion promotes an M2 macrophage phenotype and attenuates high-fat diet–induced atherosclerosis.

### Materials and Methods

**Materials and Methods**

Materials and Methods are available in the online-only Data Supplement.

**Mice**

The Cre-loxP system was used to specifically ablate GSK3α or GSK3β expression in myeloid or hepatic cells of LDLR−/− mice. To generate mice lacking GSK3α or GSK3β in hepatic cells, LDLR−/− mice were crossed with GSK3α−/−Cre2 or GSK3β−/−Cre2 mice (resulting in LDLR−/−GSK3α−/− and LDLR−/−GSK3β−/− mice). Resultant mice were crossed with mice expressing Cre recombinase under the control of the hepatic-specific albumin promoter (Alb). This breeding strategy resulted in LDLR−/−GSK3αflox;LyzMCre+ mice, referred to as LDLR−/− myeloid-specific GSK3α knockout (LMαKO) and LDLR−/−GSK3βflox;LyzMCre+ mice, referred to as LDLR−/− myeloid-specific GSK3β knockout (LMβKO). As controls, littermates lacking expression of the Cre recombinase were used and are referred to as LDLR−/− GSK3α floxed (Ltf/f/+; LDLR−/−GSK3αflox) and LDLR−/− GSK3β floxed (Ltf/f/+; LDLR−/−GSK3βflox). All mice have a C57Bl/6 genetic background (>99.9%). Genotypes were determined by polymerase chain reaction (Figure I in the online-only Data Supplement). All mice were fed a HFD (21% milk fat, 0.2% cholesterol, Harlan Teklad, TD97363) for 10 weeks beginning at 5 weeks. GSK3α−/− mice were used for bone marrow transplantation experiments. Briefly, 5-week-old recipient mice were irradiated and then injected with bone marrow (3×10^6 cells) from donor mice. Recipient mice were then fed a HFD for 10 weeks. All mice had unlimited access to food and water and were maintained on a 12-hour light/dark cycle. All animal experiments were conducted with approval of the McMaster University Animal Research Ethics Board.

### Results

**Hepatic or Myeloid Cell–Specific GSK3α or β Knockout LDLR−/− Mice Are Viable and Develop Normally**

Tissue-specific ablation of GSK3α or GSK3β protein expression was confirmed by immunoblot (Figure 1A). LMαKO and LLβKO mice have no detectable expression of hepatic GSK3α or GSK3β protein, respectively, but retain GSK3α/β expression in all other tissues, including heart, quadriceps muscle, and brain. Similarly, LMαKO and LMβKO mice have no detectable expression of macrophage GSK3α or GSK3β protein, respectively, but retain GSK3α/β expression in all other tissues. There was no detectable compensation in the expression of the retained homolog when GSK3α−/− or GSK3β−/− was deleted in hepatic or myeloid cells (Figure 1B). All mice are viable, fertile, born at expected Mendelian frequencies, and do not display any overt phenotype. All mouse strains have similar body weights to littermate controls (Tables 1 and 2; Figure 1B in the online-only Data Supplement).

**Hepatic GSK3α or GSK3β Deletion Does Not Affect Atherosclerosis**

To determine the effect of hepatic GSK3α or GSK3β deletion on atherosclerosis at the aortic root, female LLαKO and LLβKO mice and Ltf/f/+ and Ltf/f/+ controls were placed on a HFD (21% milk fat and 0.2% cholesterol) for 10 weeks beginning at 5 weeks. Metabolic parameters, including plasma cholesterol, triglyceride, glucose, liver weight, and adipose weight, were not altered in HFD-fed LLαKO and LLβKO mice relative to controls (Table 1). Analysis of atherosclerotic lesions at the aortic root revealed no change in lesion area, lesion volume, or necrotic core volume in LLαKO and LLβKO mice relative to Ltf/f/+ and Ltf/f/+ controls (Figure II–IV). In III–IV in the online-only Data Supplement). Hepatic GSK3α or GSK3β deletion did not affect plasma interleukin (IL)-6, tumor necrosis factor-α, or IL-10 levels (Figure II–IV). Similar effects were observed in male mice (Figure III in the online-only Data Supplement). These data suggest that modulation of hepatic GSK3α or β activity does not affect atherosclerosis.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tbody>
<tr>
<td>GSK3</td>
<td>glycogen synthase kinase-3</td>
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<tr>
<td>HFD</td>
<td>high-fat diet</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
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<tr>
<td>LLαKO</td>
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<td>Ltf/f/+</td>
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Nonstandard abbreviations and acronyms are defined in the online-only Data Supplement.
Hepatic GSK3α or GSK3β Deletion Does Not Affect Hepatic Lipid Content
GSK3α/β signaling plays an important role in the regulation of cellular lipid metabolism.12-14,20,21 However, the homolog-specific functions of GSK3α and GSK3β in hepatic lipid metabolism have not been determined. Therefore, we analyzed lipid metabolism in the liver of HFD-fed LLαK0 and LLβK0 mice. Relative to Ldf/fl and Ldf/fl controls, LLαK0 and LLβK0 mice do not display altered liver neutral lipid, cholesterol, or triglyceride content (Figure 2A–2D and 2F–2I). Moreover, hepatic GSK3α or GSK3β deletion did not result in altered plasma lipid profiles (Figure 2E and 2J). Finally, liver expression of genes involved in lipid homeostasis, including SREBP1c, SREBP2, HMGCoA, FAS, SR-A, SR-B1, ABCA1, and ABCG1, was unaffected by hepatic GSK3α or GSK3β deletion in mice fed a HFD (Figure IV in the online-only Data Supplement).

Myeloid GSK3α Deletion Attenuates Atherosclerosis
To determine the role of GSK3α in myeloid cells during atherosclerosis, LMrK0 and Ldf/fl control mice were placed on a HFD. After 10 weeks of HFD feeding, myeloid cell ablation of GSK3α did not significantly alter plasma cholesterol, triglyceride, or glucose concentrations or affect liver or adipose weight (Table 2). Furthermore, myeloid GSK3α deletion did not affect hepatic lipid content or plasma lipid profiles relative to controls (Figure VA–VE in the online-only Data Supplement). LMrK0 mice did, however, have significantly reduced atherosclerotic lesion area, lesion volume, and necrotic core volume relative to Ldf/fl controls (Figure 3A–3D). Myeloid GSK3α deletion also reduced lesion collagen content, fibrous cap thickness, and smooth muscle content, suggestive of less complex and less advanced lesions (Figure VIA–VIE in the online-only Data Supplement). Recent evidence suggests that

### Table 1. Metabolic Parameters of LLαK0, LLβK0, and Control Mice (n=12)

<table>
<thead>
<tr>
<th>Metabolic Parameter</th>
<th>Ldf/fl</th>
<th>LLαK0</th>
<th>Ldf/fl</th>
<th>LLβK0</th>
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<tr>
<td>Fasting plasma concentration, mmol/L</td>
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<td>Glucose</td>
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<td>Cholesterol</td>
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<td>Liver weight, g</td>
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<td>Body weight, g</td>
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<td>23.8±2.3</td>
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Ldf/fl indicates LDLR−/− GSK3α floxed; Ldf/fl indicates LDLR−/− GSK3β floxed; LLαK0, LDLR−/− liver-specific GSK3α knockout; and LLβK0, LDLR−/− liver-specific GSK3β knockout.

### Table 2. Metabolic Parameters of LMrK0, LMrβK0, and Control Mice (n=12)

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<th>Metabolic Parameter</th>
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<th>Ldf/fl</th>
<th>LMrβK0</th>
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<td>Fasting plasma concentration, mmol/L</td>
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<tr>
<td>Glucose</td>
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<td>7.4±1</td>
<td>6.8±1.3</td>
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<td>Triglyceride</td>
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<td>2.5±0.2</td>
<td>2.3±0.3</td>
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<tr>
<td>Adipose weight, g</td>
<td>0.24±0.12</td>
<td>0.25±0.12</td>
<td>0.23±0.11</td>
<td>0.21±0.09</td>
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<tr>
<td>Liver weight, g</td>
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<td>1.10±0.22</td>
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<td>1.14±0.31</td>
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<td>Body weight, g</td>
<td>23.3±1.8</td>
<td>24.0±1.4</td>
<td>23.8±1.7</td>
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Ldf/fl indicates LDLR−/− GSK3α floxed; Ldf/fl indicates LDLR−/− GSK3β floxed; LLαK0, LDLR−/− liver-specific GSK3α knockout; and LLβK0, LDLR−/− liver-specific GSK3β knockout.

Figure 1. Characterization of hepatic and myeloid cell-specific glycerogen synthase kinase (GSK)-3α and GSK3β knockout low-density lipoprotein receptor (LDLR)−/− mice. A, Whole tissue lysates from indicated tissues of LDLR−/− liver-specific GSK3α knockout (LlrαK0), LDLR−/− liver-specific GSK3β knockout (LlrβK0), LDLR−/− myeloid-specific GSK3α knockout (LmrαK0), and LDLR−/− myeloid-specific GSK3β knockout (LmrβK0) mice were separated by SDS-PAGE and probed for GSK3α and GSK3β protein levels in hepatic or myeloid tissue. B, Quantified GSK3α and GSK3β protein levels in liver tissue and macrophages determined by densitometry analysis. Data are shown as a fold change relative to the corresponding homolog in the same tissue of the control mice. ns indicates not significant. n=4. *P<0.05.

GSK3α/β profiles (Figure 3A–3D). Myeloid GSK3α deletion also reduced lesion collagen content, fibrous cap thickness, and smooth muscle content, suggestive of less complex and less advanced lesions (Figure VIA–VIE in the online-only Data Supplement). Recent evidence suggests that...
of HFD, LMαKO mice displayed reduced plasma levels of the proinflammatory cytokines IL-6 and tumor necrosis factor-α, and increased plasma levels of the anti-inflammatory cytokine IL-10 (Figure 3E). Similar effects were observed in male LMαKO mice (Figure VIIIA–VIIC in the online-only Data Supplement).

Whole-body GSK3α deficiency attenuates atherosclerosis in HFD-fed LDLR−/− mice.19 To investigate the role of myeloid GSK3α in an alternate model of experimental atherosclerosis, LDLR−/− mice were irradiated and transplanted with LDLR−/−GSK3α−/− or LDLR−/−GSK3α+/- (control) bone marrow. We found that atherosclerosis was attenuated in LDLR−/− mice receiving LDLR−/−GSK3α−/− bone marrow relative to controls (Figure VIIIA–VIIIC in the online-only Data Supplement). Conversely, transplantation of LDLR−/− bone marrow into LDLR−/−GSK3α−/− mice enhanced atherosclerotic lesion formation relative to control LDLR−/−GSK3α−/− mice transplanted with LDLR−/−GSK3α+/- bone marrow (Figure VIIID–VIIIF in the online-only Data Supplement). All bone marrow transplantation models displayed unaltered metabolic parameters (Table I in the online-only Data Supplement).

Myeloid GSK3β Deletion Does Not Alter Atherosclerosis

Myeloid GSK3β deletion did not affect plasma cholesterol, triglyceride, or glucose concentration or alter liver or adipose weight (Table 2). Moreover, LMβKO mice display similar levels of hepatic lipids and an unaltered plasma lipid profile relative to Lβfl/fl controls (Figure VFI–VJ in the online-only Data Supplement). At the aortic root, myeloid GSK3β deletion did not affect atherosclerotic lesion area or volume (Figure 4A–4C). LMβKO mice did, however, have reduced necrosis within their atherosclerotic lesion (Figure 4D). Myeloid GSK3β deficiency did not alter lesion fibrous cap formation (Figure 4D).

Figure 2. Hepatic and plasma lipids in female LDLR−/− liver-specific GSK3α knockout (LLαKO) and LDLR−/− liver-specific GSK3β knockout (LLβKO) mice. Representative images of hepatic tissue sections from LLαKO mice (A), LLβKO mice (F), and control mice stained with Oil-Red-O and hematoxylin. B and G, Quantification of Oil-Red-O stained area. Cholesterol (C and H) and triglyceride (D and I) levels within hepatic tissue of LLαKO, LLβKO, and control mice. E and J, Plasma lipid profiles of LLαKO, LLβKO, and control mice. n=6 to 10. *P<0.05.

Figure 3. Atherosclerosis in female low-density lipoprotein receptor (LDLR)−/− myeloid-specific Glycogen synthase kinase (GSK)-3α knockout (LMαKO) mice. A, Representative aortic root sections from high-fat diet (HFD) fed LMαKO and LDLR−/−GSK3α floxed (Lβfl/fl) littermate control mice stained with hematoxylin and eosin (H&E), Masons trichrome, or Oil-Red-O. B, Quantification of atherosclerotic lesion area at the aortic sinus and ascending aorta of LMαKO and control mice. Quantification of atherosclerotic lesion volume (C) and necrotic core volume (D), n=10 to 12. *P<0.05. E, Plasma levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-10 after 10 weeks of HFD. n=6 to 8. *P<0.05.
GSK3α Deletion Promotes M2 Macrophage Polarization

We examined the possible effects of myeloid GSK3α or GSK3β deficiency on monocyte subsets and macrophage phenotype. We first established that myeloid GSK3α or GSK3β deletion does not alter the number of monocytes in whole blood (Figure IXA in the online-only Data Supplement). Next, Cd115+/Cd11b+ monocytes were gated from whole blood of LMαKO, LMβKO, and control mice; Ly6C expression was determined by flow cytometry. Myeloid deletion of GSK3α or GSK3β did not affect the proportion of Ly6C+Ly6C− or Ly6C− Ly6C+ monocyte populations, suggesting that GSK3α/β does not play a role in the differentiation of these monocyte subsets (Figure IXB in the online-only Data Supplement).

We next investigated the effect of GSK3α or GSK3β deletion on macrophage polarization within atherosclerotic lesions. Analysis of the entire length of the lesion revealed that Mac3+ macrophages from lesions of LMαKO mice displayed decreased expression of the M1 markers, fatty acid binding protein-4, and CD36 and increased expression of the M2 markers Arginase1 and peroxisome proliferator-activated receptor γ coactivator (Pgc)-1 (Figure 5). Mac3+ cells within the atherosclerotic lesions of LMβKO mice showed no change in the expression of M1 markers and an increase in the expression of the M2 marker Pgc1, but not of the Arg1 (Figure 5).

To further investigate the role of GSK3α and GSK3β in macrophage polarization, bone marrow was isolated from LMαKO, LMβKO, and control mice. Cells were exposed to 10 ng/mL MCSF for 5 days to induce differentiation into unstimulated, M0, bone marrow–derived macrophages. Bone marrow–derived macrophages were then polarized into classical M1 macrophages by exposure to 10 ng/mL lipopolysaccharide for 6 hours or polarized into alternative M2 macrophages by exposure to 10 ng/mL IL-4 for 24 hours. Total RNA was isolated from the cells, and the transcript expression of genes suggestive of M1 or M2 polarization was determined. GSK3α-deficient macrophages treated with lipopolysaccharide had significantly decreased expression of the M1 markers tumor necrosis factor-α, IL-6, CD36, fatty acid binding protein-4, IL-1β, and IL-12 (Figure 6A). Similarly treated GSK3β-deficient macrophages had decreased expression of fatty acid binding protein-4, IL-1β, and IL-12 (Figure 6A). GSK3α-deficient macrophages had elevated levels of the M2 markers IL-10, Arg1, Fizz1, Ym1, Mgl-2, and Pgc1β, when exposed to IL-4 (Figure 6B). Similarly treated GSK3β-deficient macrophages had elevated expression of Arg1 only, relative to controls (Figure 6B).

Signal transducer and activator of transcription (STAT) proteins regulate transcription of the M1 and M2 gene programs (reviewed by Biswas and Mantovani28). In M1 polarized cells, STAT1 becomes phosphorylated and promotes the transcription of the M1 gene program.29–31 As a negative feedback mechanism, STAT3 is also phosphorylated in M1 cells and suppresses P-STAT1 activity by forming STAT3:STAT1 heterodimers.32–35 Exposing macrophages to IL-4 results in the phosphorylation of STAT6 and the transcription of the M2 gene program.36–38 We investigated the effect of GSK3α or GSK3β deletion on STAT phosphorylation/activation. Exposure of bone marrow–derived macrophages to lipopolysaccharide

![Figure 4. Atherosclerosis in female low-density lipoprotein receptor (LDLR)−/− myeloid-specific Glycogen synthase kinase (GSK)-3β knock-out (LMβKO) mice. A, Representative aortic root sections from high-fat diet (HFD) fed LMβKO and LDLR−/− GSK3β floxed (Lβ/β) littermate control mice stained with hematoxylin and eosin (H&E). Masons trichrome, or Oil-Red-O. B, Quantification of atherosclerotic lesion volume (C) and necrotic core volume (D). n=10 to 12. *P<0.05. E, Plasma levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-10 after 10 weeks of HFD. n=6 to 8. *P<0.05.](http://atvb.ahajournals.org/)

**Figure 4.** Atherosclerosis in female low-density lipoprotein receptor (LDLR)−/− myeloid-specific Glycogen synthase kinase (GSK)-3β knock-out (LMβKO) mice. A, Representative aortic root sections from high-fat diet (HFD) fed LMβKO and LDLR−/− GSK3β floxed (Lβ/β) littermate control mice stained with hematoxylin and eosin (H&E). Masons trichrome, or Oil-Red-O. B, Quantification of atherosclerotic lesion volume (C) and necrotic core volume (D). n=10 to 12. *P<0.05. E, Plasma levels of interleukin (IL)-6, tumor necrosis factor (TNF)-α, and IL-10 after 10 weeks of HFD. n=6 to 8. *P<0.05.

thickness, collagen content, or smooth muscle cell content (Figure VII–VII). After 10 weeks of HFD, the only detected difference in the plasma cytokine profile of LMβKO mice was a decrease in IL-6 with no change in tumor necrosis factor-α or IL-10 levels (Figure 4E). Similar effects were observed in male LMβKO mice (Figure VII–VII in the online-only Data Supplement).
resulted in the phosphorylation and activation of STAT1 and STAT3 (Figure 6C and 6D). GSK3α or GSK3β deletion did not alter P-Tyr701-STAT1 levels (Figure 6C and 6D). GSK3α deletion, but not GSK3β deletion, promoted P-Tyr705-STAT3 in lipopolysaccharide-treated macrophages (Figure 6C and 6D). Polarization of bone marrow–derived macrophages into M2 macrophages by IL-4 induced STAT6 phosphorylation and deletion of GSK3α, but not of GSK3β, promoted P-Tyr641-STAT6 relative to controls (Figure 6C and 6D). Together, these data suggest that myeloid GSK3α deficiency enhances the M2 macrophage phenotype through increased STAT3 and STAT6 phosphorylation and activation.

**Discussion**

Here, we present the first evidence for tissue- and homolog-specific functions of GSK3α/β in atherosclerosis. Myeloid GSK3α deletion, but not GSK3β deletion, attenuated atherosclerosis in HFD LDLR−/− mice and promoted an anti-inflammatory cytokine profile. Bone marrow from LDLR−/− (GSK3α−/−) mice enhanced atherosclerotic lesion formation when transplanted into LDLR−/−GSK3α−/− mice. Moreover, macrophage GSK3α deletion suppressed M1 polarization and promoted M2 polarization in both cultured macrophages and macrophages within the atherosclerotic lesion. Hepatic deletion of GSK3α or GSK3β did not alter liver lipid levels, plasma lipid profiles or atherosclerosis at the aortic root. Together, these data highlight a critical and specific role for myeloid GSK3α in atherosclerosis progression and macrophage phenotype.

Signaling through GSK3α/β is important in the regulation of liver glucose and lipid metabolism. Systemic inhibition of GSK3α/β improves glucose tolerance and insulin sensitivity in multiple rodent models of diabetes.39,40 We have previously shown that systemic inhibition of GSK3α/β or whole-body deletion of GSK3α attenuates hepatic steatosis.39,40 Herein, we present data that hepatocyte or myeloid cell–specific deletion of GSK3α or β does not alter plasma or liver lipid levels. Similarly, hepatocyte-specific deletion of GSK3α or GSK3β does not alter glucose or insulin metabolism, tolerance, or signaling.22,23 These observations may be explained by the
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A high degree of homology and substrate similarity between GSK3α and GSK3β suggest functional redundancy in lipid metabolism within hepatic cells. Moreover, these observations may be indicative of the complex coordination between liver endothelial cells, Kupffer cells, and hepatocytes in the regulation of liver lipid metabolism. Indeed,

Figure 6. M1 and M2 macrophage polarization of Glycogen synthase kinase (GSK)-3α or GSK3β-deficient bone marrow-derived macrophages (BMDMs). BMDMs were isolated from female LmαKO, LmβKO, and control mice and exposed to 10 ng/mL lipopolysaccharide (LPS) for 6 hours to induce M1 polarization or 10 ng/mL IL-4 for 24 hours to induced M2 polarization. Transcript expression of genes associated with M1 and M2 macrophage polarization was determined by reverse transcriptase-polymerase chain reaction. A, M1 polarization markers included tumor necrosis factor (TNF)-α, interleukin (IL)-6, CD36, fatty acid binding protein (FABP)-4, IL-1β, and IL-12. B, M2 polarization markers included IL-10, Arg1, Fizz1, Ym1, Mgl-2, and Pgc1β. C, Cell lysates were resolved by SDS-PAGE, and immunoblots were probed for P-STAT1, P-STAT3, and P-STAT6; total STAT1, STAT3, and STAT6; and β-actin. D, Densitometry analysis of STAT phosphorylation in BMDMs. n=4 to 5. *P<0.05.
depletion of Kupffer cells attenuates hepatic steatosis and improves insulin sensitivity.41–43 Together, these results suggest that functional GSK3α/β signaling in multiple cell types coordinate glucose, insulin, and lipid metabolism in the liver.

Before this study, investigations into the role of GSK3α/β in animal models of atherosclerosis used nonspecific inhibitors or whole-body genetic deletion.19–21 These studies were unable to differentiate whether GSK3α/β’s role in atherosclerosis was because of alterations in peripheral tissue, such as the liver or adipose, or local alterations within the vessel wall. In this report, we clearly show that GSK3α plays a local role in myeloid cells within the aortic wall during atherosclerosis development. In support of our results from the LMαKO mice, we observe that transplantation of LDLR−/− (GSK3α−/−) bone marrow into LDLR−/−GSK3α−/− mice restores atherosclerotic lesion formation. This is a critical observation because LDLR−/−GSK3α−/− mice transplanted with LDLR−/− bone marrow lack GSK3α expression in other cell types important to atherosclerosis progression, including endothelial cells and smooth muscle cells, and further suggests that GSK3α’s function in atherosclerosis is myeloid cell specific. However, as in liver pathology, cross talk between cell types within the lesion is important during atherosclerosis development.44 Our observations of reduced collagen deposition, fibrous cap thickness, and smooth muscle cell content in the lesions of LMαKO mice, suggestive of less complex and less advanced lesions, are consistent with this hypothesis.

Recent studies have implicated a role for GSK3α/β signaling in macrophages function.12,19,25,26 We have extended these findings and present the first evidence suggesting that GSK3α regulates macrophage, but not monocyte, polarization. Although deletion of GSK3α does not affect Ly6Chigh/int/low monocyte populations, it does shift macrophage polarization toward an M2 phenotype and away from an M1 phenotype. This shift along the macrophage spectrum toward an M2 phenotype may explain the anti-inflammatory cytokine profile and attenuated atherosclerosis observed in LMαKO mice. Indeed, deletion of STAT1, a key promoter of M1 genes, attenuates atherosclerosis, whereas IL-13 deletion inhibits M2 polarization and promotes atherosclerosis.6–8 Moreover, during regression of atherosclerotic lesions macrophages switch from an M1 phenotype to an M2 phenotype.45,46 Mhem and Mox macrophages also play functional roles in atherosclerosis progression; however, their contributions are less well characterized.

Together, our data are consistent with a model in which proatherogenic stimuli signal through macrophage GSK3α to promote lesion growth and development. The factors that lie directly upstream of GSK3α have yet to be identified. Our previous results have suggested that endoplasmic reticulum stress activates GSK3α/β via induction of the PERK pathway.12,20 Recently, the serine/threonine kinase AKT has been shown to modulate atherosclerosis and macrophage polarization.47 This is intriguing because AKT is activated by endoplasmic reticulum stress and is a known regulator of GSK3α/β. The factors lying directly downstream of GSK3α are also not known. Herein, we present evidence for STAT3 and STAT6 being targets of GSK3α signaling. Increased P-STAT3 in GSK3α-null M1 cells and increased P-STAT6 in GSK3α-null M2 cells underscore the role of GSK3α activation in shifting macrophages toward the M1 phenotype and promoting atherosclerosis. Furthermore, GSK3α/β is an established regulator of nuclear factor-κB, which also influences macrophage phenotype.17,48 The identification of the factors directly upstream and downstream of GSK3α in JAK/STAT signaling, and their effect on M1 and M2 polarization, is a critical next step.

The therapeutic potential of small molecule inhibitors of GSK3α/β has been limited by our lack of understanding of its homolog-specific functions. Our results suggest that specific targeting of GSK3α may have many clinical advantages as an antiatherogenic therapy. First, modulating specific macrophage subpopulations in the vessel wall may alter local macrophage behavior while retaining systemic functions. Furthermore, the inhibition of GSK3α specifically would be predicted to have fewer side effects because GSK3β signaling would be maintained.

In summary, we present the first evidence for tissue- and homolog-specific functions of GSK3α/β in atherosclerosis. We demonstrate that myeloid cell GSK3α signaling regulates atherosclerosis development and macrophage polarization.

Acknowledgments
We thank Dr Bradley Doble (McMaster University) and Dr Jim Woodgett (University of Toronto) for the generous gift of the floxed-GSK3α, floxed-GSK3β, and GSK3α−/− mice. We also thank Dr Bernardo Trigatti, Omid Dadoo, and Mark Fuller (McMaster University) for technical assistance.

Sources of Funding
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Disclosures
None.

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attend GSK-3α and atherosclerosis
vph.2010.09.004.
CIRCULATIONAHA.107.696922.
PLOS One
96. doi: 10.1038/ni.1937.
Neyrinck AM, Tape HS, Gevers V, Declerck B, Delzenne NM. Inhibition of Kupffer cell activity induces hepatic triglyceride synthesis


**Significance**

Despite the wide use of statins, the clinical consequences of atherosclerosis, myocardial infarction, and stroke remain the leading causes of death globally. Here, we present a novel and intriguing antiatherogenic therapeutic target, GSK3α. We present evidence describing both homolog- and tissue-specific functions of GSK3α and GSK3β in multiple animal models of atherosclerosis and in both sexes. Interestingly, deficiency of myeloid GSK3α, but not of GSK3β, attenuates atherosclerosis and reduces lesion complexity. Clinically, inhibiting GSK3α/β has been shown to reduce the instances of MI and stroke in epileptics; however, targeting the GSK3α homolog specifically may provide greater therapeutic benefit. A GSK3α-specific inhibitor would reduce atherosclerosis while retaining GSK3β activity and therefore would predict to have fewer side effects. Moreover, inhibition of GSK3α seems to alter leukocyte subpopulations within the aortic wall while retaining systemic leukocyte function.
Deletion of Myeloid GSK3α Attenuates Atherosclerosis and Promotes an M2 Macrophage Phenotype

Cameron S. McAlpine, Aric Huang, Abby Emdin, Nicole S. Banko, Daniel R. Beriault, Yuanyuan Shi and Geoff H. Werstuck
Deletion of Myeloid GSK3α attenuates atherosclerosis and promotes an M2 macrophage phenotype

McAlpine et al. ATVB, 2015

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Supplemental Material

Supplementary Table I. Metabolic parameters of bone marrow transplanted mice fed a HFD. n=5

<table>
<thead>
<tr>
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<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;GSK3α&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;GSK3α&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>LDLR&lt;sup&gt;−/−&lt;/sup&gt;GSK3α&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Fasting Plasma Concentration (mmol/L)</td>
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<tr>
<td>Glucose</td>
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<td>9.9±1.2</td>
<td>8.5±0.8</td>
<td>8.5±0.4</td>
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<td>Cholesterol</td>
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<td>29.7±4.2</td>
<td>32.2±2.4</td>
<td>25.3±2.5</td>
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<td>Triglyceride</td>
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<td>0.24±0.03</td>
<td>0.25±0.05</td>
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<td>Liver weight (g)</td>
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<td>0.99±0.03</td>
<td>0.90±0.05</td>
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## Supplementary Table II. RT-PCR Primer Sequences.

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Supplementary Figure I. PCR and body weight analysis of mouse models. (A) Genotyping of LLαKO, LLβKO, LMαKO and LMβKO mice. Lane (a) indicates LDLR−/− (~320bp band), lane (b) indicates GSK3αfl/fl (750bp band) or GSK3βfl/fl (685bp band), and lane (c) indicates expression of the appropriate Cre recombinase (100bp band for Alb Cre and 700bp + 350bp bands for LyzCre+ and wildtype respectively) (B) Body weight analysis of female mice on a HFD. n=8-9
Supplementary Figure II. Atherosclerosis in female LLαKO and LLβKO mice. Representative aortic root sections from HFD fed LLαKO and Lαfl/fl control mice (A) or LLβKO and Lβfl/fl control mice (F) stained with hematoxylin and eosin (H&E), Mason’s Trichrome or Oil Red O. Quantification of atherosclerotic lesion area at the aortic sinus and ascending aorta of LLαKO mice (B), LLβKO mice (G) and corresponding controls. Quantification of atherosclerotic lesion volume and necrotic core volume of LLαKO (C and D) and LLβKO (H and I) mice and corresponding controls. n=10-12. (E and J) Plasma levels of IL-6, TNFα and IL-10 after 10 weeks of HFD. n=5-8
Supplementary Figure III. Atherosclerosis in male LLαKO and LLβKO mice. Representative images of hematoxylin and eosin stained aortic root sections from male LLαKO (A), LLβKO (D) and control mice fed a HFD for 10 weeks. Quantification of atherosclerotic lesion area beginning at the aortic root (B and E) and lesion volume (C and F) of male mice. n=5
Supplementary Figure IV. Metabolic gene expression in liver of female LLαKO and LLβKO mice. Transcript expression of lipid metabolism genes in the liver of LLαKO, LLβKO and littermate controls fed a HFD. Genes include sterol regulatory element binding protein (SREBP)1c, SREBP2, HMGCoA, fatty acid synthase (FAS), scavenger receptor (SR)-A, SR-B1, ABCA1 and ABCG1. n=8
Supplementary Figure V. Hepatic lipids in female LMαKO and LMβKO mice. Representative images of hepatic tissue stained with Oil Red O and Hematoxylin from LMαKO (A), LMβKO (F) and control mice. Quantification of Oil Red O stained area in LMαKO (B), LMβKO (G) and control mice. Cholesterol (C and G) and triglyceride (D and H) levels within hepatic tissue of LMαKO, LMβKO and control mice. Plasma lipid profiles of LMαKO (E), LMβKO (J) and control mice. n=5-10
Supplementary Figure VI. Lesion characterization in female LMaKO and LMBKO mice. Aortic root sections from LMaKO, LMBKO and control mice stained with Maisons trichrome (A and F). Fibrous cap thickness (B and G) and collagen content relative to lesion area (C and H) were quantified. (D and I) Aortic root sections from LMaKO, LMBKO and control mice stained with the smooth muscle cell marker alpha actin. (E and J) Alpha actin staining intensity within the atherosclerotic lesions was quantified. n=6 *p<0.05
**Supplementary Figure VII.** Atherosclerosis in male LMαKO and LMβKO mice. Representative images of hematoxylin and eosin stained aortic root sections from male LMαKO (A), LMβKO (D) and control mice fed a HFD for 10 weeks. Quantification of atherosclerotic lesion area beginning at the aortic root (B and E) and lesion volume (C and F) of male mice. n=5, *p<0.05
Supplementary Figure VIII. Atherosclerosis in female bone marrow transplanted mice. (A) Representative images of aortic root sections stained with hematoxylin and eosin from LDLR<sup>−/−</sup> mice transplanted with LDLR<sup>−/−</sup> or LDLR<sup>−/−</sup>GSK3α<sup>−/−</sup> bone marrow and fed a HFD for 10 weeks. (B) Quantification of lesion area at the aortic root. (C) Quantification of lesion volume. (D) Representative images of hematoxylin and eosin stained aortic root sections from LDLR<sup>−/−</sup>GSK3α<sup>−/−</sup> mice transplanted with LDLR<sup>−/−</sup>GSK3α<sup>−/−</sup> or LDLR<sup>−/−</sup> bone marrow and fed a HFD for 10 weeks. (E) Quantification of lesion area at the aortic root. (F) Quantification of lesion volume. n=5, *p<0.05
Supplementary Figure IX. Ly6C expression on monocytes from female LMAKO and LMBKO mice. (A) Number of monocyte in the blood of LMAKO, LMBKO and control mice fed a HFD for 5 weeks. (B) Cd115+Cd11b+Ly6C<sup>lo</sup>, Cd115+Cd11b+Ly6C<sup>int</sup> and Cd115+Cd11b+Ly6C<sup>hi</sup> monocyte levels in the blood of LMAKO, LMBKO and control mice fed a HFD for 5 weeks. n=8-10
Supplementary Figure X. Representative images of control Igg immunoflorescent staining and specific antibody staining.
Deletion of Myeloid GSK3α attenuates atherosclerosis and promotes an M2 macrophage phenotype

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Supplemental Materials and Methods

Mouse models

All animal experiments were conducted with approval of the McMaster University Animal research Ethics Board. LDLR<sup>-/-</sup> mice (B6.129S7-Ldlr<sup>tm1Her</sup>/J), AlbCre mice (B6N.Cg-TgAlb-cre<sup>21Mgn</sup>/J) and LyzMCre (B6.129P2-Lyz2<sup>tm(cre)If0</sup>/J) mice were purchased from Jackson Labs. Floxed GSK3α, floxed GSK3β and GSK3α<sup>-/-</sup> mice were a generous gifts from Dr. Bradley Doble (McMaster University) and Dr. Jim Woodgett (University of Toronto).<sup>1-4</sup> All mice were backcrossed >10 times onto a C57Bl6 background. Genotype was determined by PCR (Supplementary Figure 1). Tissue specific GSK3α or GSK3β knockout LDLR<sup>-/-</sup> mice were placed on a high fat diet (HFD) containing 21% milk fat, 0.2% cholesterol (42% kcal from fat)(Harlan Teklad, TD97363) at 5 weeks of age and sacrificed at 15 weeks of age. For bone marrow transplantation experiments, 5 weeks old recipient mice were irradiated with 12Gy of γ-irradiation from a <sup>137</sup>C source using a Gammacel 3000 small animal irradiator. Irradiated mice received bone marrow (3X10<sup>6</sup> cells) injected by i.v. and were left to recover for 4 weeks before being switched to a HFD for 10 weeks (from 9 to 19 weeks of age). All mice had unlimited access to food and water. Body weights were monitored and fasted blood glucose and lipid levels were quantified as previously described.<sup>5</sup> Pooled plasma from at least 5 animals of each group was fractioned using fast performance liquid chromatography with the FRAC-950 FPLC (Amersham Pharmacia Biotech) and cholesterol concentration was measured using the infinity cholesterol reagent (Thermo Scientific). Plasma IL-6, TNFα and IL-10 was quantified by ELISA (eBioScience). Monocyte concentration in whole blood was determined using a Hemavet 950 Multi Species Hematology System (Drew Scientific). Plasma and tissue cholesterol and triglyceride levels were determined using infinity reagent (Thermo Scientific).

Tissue collections and analysis

After 10 weeks of HFD feeding mice were anesthetized (isoflourane) and harvested at 15 weeks (tissue specific knockout mice) or 19 weeks (bone marrow transplant mice) of age. Vasculature was flushed with saline and perfusion fixed with 10% neutral buffer formalin. Hearts, aortas and liver were collected for further analysis. Hearts and aortas were imbedded in paraffin and serial 5μm sections of the aortic root were collected. Sections were stained with Harris hematoxylin and eosin (Sigma) and Masons Trichrome (Sigma). On a separate subset of mice, hearts, aortas and liver were collected in optical cutting temperature (OCT) compound (Tissue-Tek) and frozen. OCT imbedded frozen tissue was serial sectioned at 10μm and stained with Oil Red O and Mayer’s Hematoxylin (Sigma). Images were collected using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera and lesion area was quantified using Image J 1.43M software as previously described.<sup>6</sup> Briefly, for atherosclerotic lesion area, volume and necrotic core volume paraffin imbedded sections stained with H&E were used. Sections 60μm apart spanning the entire length of the lesion were used. Fibrous cap thickness was determined throughout the length of the lesion using ImageJ software. Average fibrous cap thickness is
presented. Lesion collagen content was determined by quantifying collagen stained area using ImageJ software and is presented relative to total lesion area.

**Immunoblot**

Total protein lysates were prepared from flash frozen tissue solubilized in 4x SDS-PAGE sample buffer (0.5M Tris-HCl pH6.8, glycerol and 10% SDS) and quantified by Bradford assay. 30µg of total protein was separated by SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes. Membranes were incubated with antibodies overnight at 4˚C. Antibodies used include GSK3α/β, P-701-STAT1, STAT1, P-705-STAT3, STAT3, STAT 6 (Cell Signaling) and P-641-STAT6 (BD Biosciences). Membranes were then exposed to the appropriate horseradish peroxidase secondary antibody (Life Technologies) for 1 hr at room temperature and developed using Immobilon Western Chemiluminescent horseradish peroxidase substrate (Millipore). Densitometry analysis of bands was conducted using Image Lab software (Invitrogen).

**Bone marrow derived macrophage isolation and polarization**

Bone marrow was isolated from tibias and femurs of mice and cultured in DMEM (Life Technologies) containing 10% fetal bovine serum. Bone marrow was exposed to 10ng/ml mouse macrophage colony stimulating factor (MCSF) for 5 days producing unstimulated (M0) bone marrow derived macrophages (BMDMs). BMDMs were then polarized into M1 macrophages by exposure to 10ng/ml LPS for 6 hours or M2 macrophages by exposure to 10ng/ml IL-4 for 24 hours.

**Gene expression**

Total RNA was isolated from BMDMs or hepatic tissue using the RNeasy Mini Kit (Qiagen). RNA concentration and purity was determined by measuring the absorbance at 260nm/280nm. RT-PCR was performed using 2µg of total RNA transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Amplification of PCR products was carried out using a GeneAmp 7300 sequence detection system (Applied Biosystems) and SYBR Green qPCR SuperMix (Invitrogen). PCR reactions were performed in technical triplicates and biological quadruplets using primers sequences indicated in Supplemental Table II. RT-PCR results were analyzed using Data Assist 3.0 software (Applied Biosystems).

**Flow cytometry**

Blood was collected via the tail vein and Ly6C expression was determined using a BD LSR II flow cytometer. Using forward scatter and side scatter plots, red blood cell and dead cells were excluded from analysis. CD115+ and CD11b+ monocytes were identified using PE- and FITC-conjugated antibodies (BD Pharmingen). Ly6C expression was then determined in the monocyte population using an APC-conjugated antibody (BD Pharmingen).

**Immunoflorescence**

Paraffin imbedded serial sections (4µm) were immunostained using primary antibodies against Mac3 (BD Transductions), αactin (Santa Cruz), FABP4 (Cell Signaling), CD36 (Santa Cruz), Arg1 (Santa Cruz) and Pgc1 (Santa Cruz). Serial sections were stained with pre-immune IgG, in place of primary antibodies, to control for non-specific staining (Supplementary Figure X). Appropriate secondary antibodies (BD transductions) conjugated to a florophore were used for detection. Images were collected using a Leitz LABORLUX S microscope connected to a DP71 Olympus camera. Immunofluorescent staining intensity was quantified using Image J 1.43M.
software. Briefly, 12 aortic sections from each animal (n=5-6 mice per group) representing the entire length of the lesion were stained and imaged. Mac3+ stained area was excised from each image and FABP4, CD36, Arg1 and Pgc1 staining intensity above background within the Mac3+ area of the atherosclerotic lesion was determined over a fixed threshold. For α-actin staining the entire lesion area was excised from the image and similarly quantified. The staining intensity of the 12 aortic sections from each animal was averaged to provide a staining intensity for each animal. Data shown represent average staining intensity for each animal within the group.

Statistical analysis

All data is expressed as mean±SD of independent biological experiments. The number of biological replicates is indicated in the figure legends. An unpaired Student t test or a 1-way ANOVA test was used to determine statistical significance. A value of P<0.05 was considered statistically significant.

Supplementary References